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BACTERIA CONCERNED IN THE PRODUCTION OF THE CHARACTERISTIC FLAVOR IN CHEESE OF THE CHEDDAR TYPE

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BACTERIA CONCERNED IN THE PRODUCTION OF THE CHARACTERISTIC FLAVOR IN CHEESE OF THE CHEDDAR TYPE[1]

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In a previous publication (Hastings, Evans, and Hart, 1912)[2] a summary of the knowledge concerning the bacteriology of Cheddar cheese was presented together with the results that were obtained by the authors in an effort to extend the knowledge concerning the flora of this type of cheese. It was shown, as had previously been demonstrated by numerous investigators, that *Bacterium lactis acidi* is an important factor in the making and ripening of Cheddar cheese. The lactic acid formed aids in the curdling of the milk by the rennet, and the acid formed in the curd assists in the expulsion of whey therefrom. The combination of the acid with the paracasein so changes the nature of the curd that the pieces readily coalesce or "mat." The lactic acid also activates the pepsin of the rennet extract, enabling it to act on the paracasein, with the formation of soluble products. The acid reaction of the cheese is maintained during the ripening period and thereafter, thus inhibiting the development of putrefactive bacteria which otherwise would soon destroy the commercial value of the cheese.

It was further shown that lactic bacilli[3] occur in all Cheddar cheese in numbers approximating those of the ordinary lactic bacteria, *B. lactis acidi*, and that coccus forms are also of constant occurrence.

At the beginning of the ripening period the ratio between *Bacterium lactis acidi* and lactic bacilli can, in a general way, be expressed as 99 to 1. This ratio gradually changes as the ripening progresses until in cheese 3 to 6 months old the ratio is reversed. It is apparent that the larger part of the growth of the *B. casei* group must take place after the fermentation

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[1] Work of Department of Agriculture in cooperation with Wisconsin Agricultural Experiment Station.

[2] Bibliographic citations in parentheses refer to "Literature cited," pp. 191-192.

[3] This group of lactic-acid-forming organisms appears in the literature under various names, the most common being "lactic bacilli," "*Bacterium bulgaricum*" or "*Bacillus bulgaricus*," and "*Bacterium casei*." The name "*Bacterium casei*" will be used in this article.

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of the sugar in the cheese by *B. lactis acidi*. Their continued persistence in large numbers must be due to continued growth for a considerable period or else to their greater resistance to the environment. But little definite information was presented in the bulletin mentioned concerning the occurrence of coccus forms, because of the confusion then existing in the differentiation of some varieties of this group from the *B. lactis acidi* group.

It was recognized that we were not dealing with specific organisms but rather with great groups. In making cheese of the Cheddar type from pasteurized milk it was found that the typical flavor did not develop. The milk, after pasteurization, always contained organisms of the *Bacterium lactis acidi* group, of the *B. casei* group, and coccus forms, and there was added to the milk a starter containing an organism of the *B. lactis acidi* group. In order to obtain a more complete knowledge concerning the biological factors present in Cheddar cheese and their rôle in the development of flavor and possibly to determine the cause of lack of flavor in cheese from pasteurized milk, a more detailed study has been made of cheese both from raw and from heated milk, with the results as presented in this paper.

In continuation of the above-mentioned work, bacteriological analyses of many more cheeses have been made in order to determine more fully the distribution of the groups in ripening cheese; and a detailed study of the pure cultures obtained has been carried out along new lines with the view of correlating the presence of certain types with desirable or undesirable flavor production.

METHODS OF EXAMINATION

In the previous work the pure cultures were obtained by isolation from lactose-agar plate cultures and from dilution cultures made in small flasks of sterile milk. These milk cultures were inoculated from a dilution of a cheese emulsion, the dilution increasing from flask to flask by a ratio of 10. The above methods have been modified in the present study. The dilution cultures were made in milk to which was added 1 gram of peptone, 1 gram of dextrose, and 200 c. c. of water per liter. This was found to be more favorable than plain milk for the development of some of the cheese organisms. An effort was made to obtain two pure cultures for study from each series of dilution cultures; viz, the predominating organism of the combined *Bacterium lactis acidi* and coccus groups and the predominating organism of the *B. casei* group. The former was obtained by plating the culture from the highest dilution of the cheese which shows a growth after two days' incubation; the latter was obtained after three weeks' incubation. The flask cultures were then titrated to determine the highest dilution which contained *B. casei*. All milk cultures which titrated more than 1.15 per cent of acidity were further examined for the *B. casei* group. The long incubation usually killed the other groups of cheese organisms, so that a subinoculation into tubes of litmus milk containing dextrose and peptone resulted in the growth of the lactic bacilli alone. The culture was then purified by plating.

The plate cultures of the cheese dilutions were made in casein agar (Ayers, 1911), to which 1 per cent of dextrose was added; they were incubated at 37° C. for two days and then kept at room temperature for four days. In order to obtain the percentage of the various cheese organisms which develop upon the casein-dextrose-agar plates, a portion containing 10 colonies was circumscribed, and the inclosed colonies were fished off into litmus milk. When growth appeared in the milk cultures a microscopic examination was made. Those cultures from a single plate which caused the same changes in litmus milk and were of the same morphology were considered of the same variety. Representatives of every variety were studied in detail and referred to their respective places, according to the classification given below.

This method of studying only 10 colonies from the plate cultures at one analysis and then reducing this number for more detailed study to a single representative of those cultures which are similar in their morphology and their action on litmus milk was intended to give a broad view of the flora of cheese rather than a detailed one. A more intensive study of the cheeses selected for analysis would necessarily reduce the number which could be examined. Inasmuch as the flora of normal Cheddar cheese will differ greatly in a detailed study, the methods used, which allow the examination of a large number of cheeses and give a rough picture of the cheese flora, were considered the best adapted to increase our knowledge in its present stage.

FERMENTATION TESTS FOR THE CLASSIFICATION OF CHEESE ORGANISMS

The cultural characteristics, morphology, and the few biochemical reactions which are ordinarily considered in classification of bacteria were found to be inadequate traits for distinguishing one variety of cheese organism from another of the same group. Therefore the system of classification based upon the fermentation of various test substances which has been used by Gordon (1905) and other English investigators and by Winslow (1912) and his colleagues for the classification of the Coccaceæ and which was also used by Rogers and Davis (1912) for classifying the lactic-acid bacteria of the *Bacterium lactis acidi* type was adopted, with modifications suited to the problem in hand, for the classification of cheese organisms.

A sugar-free broth was made of 10 grams of compressed yeast, 10 grams of peptone, and 5 grams of dibasic potassium acid phosphate per liter of water. To this mixture was added 1 per cent of the test substance. At first inoculations were made into dextrose, lactose, galactose, sucrose, salicin, mannit, glycerin, inulin, starch, and raffinose broths with all the groups of cheese organisms. After many cultures had been submitted to these tests it was found that some of the substances had no differential value. All of the cultures produced acidity from some of the substances, while none of the cultures, or exceedingly few, produced acidity from other substances. Moreover, not all of the test substances which proved of value in differentiating into varieties the members of the coccus and *Bacterium lactis acidi* groups were equally valuable in the study of the *B. casei* group.

After a preliminary study of 250 cultures of the cheese organisms on the 10 test substances with quantitative determinations of the amount of acid produced in the dextrose broth, some of the substances were discarded. The study was continued on 5 of the test substances differing for the various groups of organisms.

It now includes approximately 1,000 cultures of cheese organisms isolated from 37 different raw-milk cheeses, which represent every stage of the ripening period. The test for acid was made after 10 days' incubation at 37° C. Small squares of alkaline litmus paper were placed in the cultures. If the color in several cultures of positive and negative reactions is compared this method will detect very slight increases in acidity.

This method of testing the production of acid with litmus paper differs from that of other investigators who have made use of the fermentation tests for the classification of bacteria. These investigators have determined by titration the amount of acid produced in the various broths and have considered these data also in their classification. But in any work of the nature of the problem under discussion it is important that the first stages should be a comprehensive survey of all of the factors which may influence the final result rather than a more intensive study of any one or more factors which attract attention at first sight. The methods which have been followed in the classification of cheese organisms were chosen with the purpose of excluding as much as possible of the routine work, which appeared to be of minor importance for this study in its present stage, in order to extend it over a larger number of samples of cheese. For this reason the fermentable substances employed for the tests were reduced to the smallest number which appeared adequate for the differentiation. And the titration of the acid produced, which in the preliminary study was made in the dextrose broth alone, was entirely omitted in the major part of the study. Rogers and Davis (1912) have adopted an increase of 1 per cent of normal acid as the division between fermentation and nonfermentation. In the case of lactose, salicin, sucrose, mannit, and inulin the reddening of alkaline litmus paper gives practically the same results as far as the division into fermenters and nonfermenters is concerned, for in broths containing these substances the reaction is almost always negative or decidedly positive, with the development of a considerable acidity. If there is a development of acidity in glycerin, the reaction is often slight. The titration of a number of such cultures showed that the litmus paper will detect increases of acidity as low as 0.5 per cent; these were recorded as positive. Hence, in the comparatively few instances in which the development of acid is slight the results may sometimes be recorded as positive in this study, where other investigators would have recorded them as negative.

The amount of acid produced is regarded as unimportant in the classification of the cheese organisms. The consideration of degrees of acidity would greatly complicate a system which, if it is to be valuable in the study of the cheese problem, should rather be made as simple as possible.

CLASSIFICATION OF THE BACTERIUM LACTIS ACIDI AND COCCUS GROUPS

In the present study an especial effort has been made to differentiate the *Bacterium lactis acidi* and coccus groups. Since they were submitted to the same tests, the classification of these groups will be considered together. The organic substances which were adopted as the most useful test substances for the coccus and *B. lactis acidi* groups are lactose, salicin, mannit, sucrose, and glycerin. The morphology, the growth on plain agar slopes, and the growth in litmus milk were also considered.

Cultures in which some or all of the cells are elongated and in pairs and which curdle litmus milk with the reduction of the litmus characteristic for *Bacterium lactis acidi* were classed in that group. The Streptococcus group includes all cultures in which the cells are spherical and arranged in pairs or in chains. The cultures of this group do not give the reduction of litmus which is characteristic for the *B. lactis acidi* group. Most of the cultures of the Micrococcus group produce a heavy growth upon the agar slope, which is often of some shade of yellow. They are the members of the Coccaceæ which divide in two planes; consequently the cells appear in pairs, fours, or bunches.

In Table I the sign ± indicates that individual cultures of the given variety may produce acid from the substance in question; or they may fail to produce acid. The test substances are arranged in the table according to their order of availability. For example, lactose is fermented by the greatest number of cultures, and mannit is fermented by the least number of cultures of both groups of the Coccaceæ.

TABLE I.—*Classification of the groups of cheese organisms*

BACTERIUM LACTIS ACIDI

Variety No.	Production of acidity in broth containing—				
	Lactose.	Salicin.	Mannit.	Sucrose.	Glycerin.
a	+	−	−	−	−
b	+	+	−	−	−
c	+	+	−	+	−
d	+	±	+	±	−

TABLE I.—*Classification of the groups of cheese organisms*—Continued

STREPTOCOCCUS

Variety No.	Production of acidity in broth containing—				
	Lactose.	Salicin.	Sucrose.	Glycerin.	Mannit.
a	–	–	–	–	–
b	+	–	±	±	–
c	+	+	±	±	–
d	+	±	±	±	+

MICROCOCCUS

Variety No.	Production of acidity in broth containing—				
	Lactose.	Sucrose.	Glycerin.	Salicin.	Mannit.
a	+	±	±	–	–
b	+	±	±	+	–
c	+	+	–	–	+

All organisms which have been found constantly in cheese are included in the four groups *Bacterium lactis acidi*, Streptococcus, and Micrococcus groups, together with the *B. casei* group, which will be discussed later. The different varieties of all four groups produce acid in milk. Spore-forming organisms occur in very small numbers or are entirely absent. The same thing is true of liquefying organisms. Whenever they are present in sufficient numbers to have an appreciable influence in the ripening, it is to the detriment of good flavor.

In the classification of the *Bacterium lactis acidi* group, variety "a" corresponds to group "A" in Rogers and Davis's classification of the lactic-acid bacteria (1912). Varieties "b," "c," and "d" are all included in their group "B," which also includes other varieties which were not found among the cheese organisms and are therefore not mentioned in this work.

CONSTANCY OF REACTION OF THE CULTURES OF THE BACTERIUM LACTIS ACIDI AND COCCUS GROUPS TO THE TEST SUBSTANCES

It is obvious that the accuracy of this method, in so far as it is a guide for the classification of an individual culture, depends on the constancy of the reactions to the tests. It is the opinion of those who have made a comprehensive study of this method that the fermentation reactions on the test substances are on the whole remarkably constant for any given strain, and therefore they are of value in classification. Representative cultures of the varieties of the coccus and the *Bacterium lactis acidi* groups have been maintained on agar slopes, with transfers made every month or six weeks. The constancy of the reactions of these cultures on the five organic substances has been tested, and the results on salicin, mannit, sucrose, and glycerin are given in Table II. The results on lactose are omitted, because they are always positive.

TABLE II.—*The constancy of the Bacterium lactis acidi and coccus groups in their action upon test substances*

BACTERIUM LACTIS ACIDI

No. of culture.	Production of acid in broth containing—														
	Salicin.					Mannit.			Sucrose.			Glycerin.			
	First test.	Age of culture at time of second test.	Second test.	Age of culture at time of third test.	Third test.	First test.	Second test.	Third test.	First test.	Second test.	Third test.	First test.	Second test.	Third test.
		Months.		*Months.*										
1	−	8	−	12	−	−	−	−	−	−	−	−	−	−
2	−	3	+	......	......	−	+	......	−	−	......	−	−	......
3	−	9	+	12	+	−	+	+	−	−	−	−	−	......
4	+	1	+	......	......	−	−	......	−	−	......	−	−	......
5	+	4	+	8	+	−	−	+	−	−	−	−	−	−
6	−	4	+	8	+	+	+	+	−	−	−	−	−	−
7	+	4	+	8	+	+	+	+	−	−	−	−	−	−
8	+	2	+	......	......	+	+	......	+	+	......	−	−	......

STREPTOCOCCUS

No. of culture.	Production of acid in broth containing—													
	Salicin.					Sucrose.			Glycerin.			Mannit.		
	First test.	Age of culture at time of second test.	Second test.	Age of culture at time of third test.	Third test.	First test.	Second test.	Third test.	First test.	Second test.	Third test.	First test.	Second test.	Third test.
		Months.		*Months.*										
9	−	10	−	14	−	−	+	+	−	−	−	−	−	−
10	−	10	−	14	−	−	−	+	−	−	+	−	−	−
11	−	6	−	......	......	+	+	......	−	−	−	−	−	−
12	−	5	−	......	......	+	+	......	+	+	......	−	−	......
13	−	9	−	......	......	+	+	......	+	+	......	−	−	......
14	+	9	+	12	+	−	−	−	−	−	−	−	−	−
15	+	4	+	8	+	−	−	−	+	+	+	−	−	−
16	+	4	+	8	+	+	+	+	−	−	−	−	−	−
17	+	5	+	......	......	+	+	......	−	−	......	+	+	......
18	−	6	−	9	−	+	+	+	+	+	+	+	+	+
19	+	5	+	9	+	−	−	−	+	−	−	+	+	+
20	+	4	+	8	+	+	+	+	+	+	+	+	+	+

TABLE II.—*The constancy of the Bacterium lactis acidi and coccus groups in their action upon test substances*—Continued

MICROCOCCUS

No. of culture.	Production of acid in broth containing—													
	Sucrose.					Glycerin.			Salicin.			Mannit.		
	First test.	Age of culture at time of second test.	Second test.	Age of culture at time of third test.	Third test.	First test.	Second test.	Third test.	First test.	Second test.	Third test.	First test.	Second test.	Third test.
		Months.		*Months.*										
21	–	8	–	11	+	–	–	–	–	–	–	–	–	–
22	–	10	+	14	–	–	–	+	–	–	–	–	–	–
23	+	9	+	13	+	–	–	–	–	–	–	–	–	–
24	+	9	+	13	+	+	–	–	–	–	–	–	–	–
25	+	6	+	9	+	–	–	–	+	–	–	–	–	–
26	–	6	–	9	+	+	–	–	+	+	+	–	–	–
27	+	4	+	8	+	+	–	–	+	+	+	–	–	–

In the *Bacterium lactis acidi* group there is a tendency for those cultures which have a low ability for fermentation to increase that ability when cultivated upon the plain agar slopes. This appears to be a change in the physiological properties of the cultures when grown under artificial conditions, for every variation which occurred in this group was an increased ability to attack the more difficultly fermentable substances. Thus, cultures 2 and 3, which at the time the first test was made fermented only lactose and were classified as variety "a," in later tests fermented also salicin and mannit and would therefore be classified as variety "d." Also, culture 5 acquired the ability to ferment mannit, and culture 6 acquired the ability to ferment salicin.

The Streptococcus cultures all remained constant in their action upon lactose, salicin, and mannit. Cultures 9 and 10 acquired the ability to ferment sucrose, and culture 10 also acquired the property of fermenting glycerin. Culture 19 lost the power of fermenting glycerin. The Micrococcus cultures also showed some inconstancy upon sucrose and glycerin, and one culture, 25, varied in its action on salicin. On the whole, the reactions are sufficiently constant to be considered valuable in the differentiation of cheese organisms.

CLASSIFICATION OF THE BACTERIUM CASEI GROUP BY BIOCHEMICAL TESTS

The study of a number of cultures of the *Bacterium casei* group isolated from Cheddar cheese showed that there is a great variation in the amount of acidity the different cultures are able to produce in milk. It has been found (Currie, 1911) also that the cultures differ in the rotatory power of their acids. It was evident that different strains of this group were present in cheese, and therefore it was desirable to classify them, in order to study the significance of the various strains in cheese ripening. Accordingly the biochemical tests which were used for the differentiation of the other cheese organisms were adopted also for the classification of this group.

The preliminary study upon the 10 original test substances showed that lactose, salicin, sucrose, mannit, and inulin have some value in the differentiation of this group. The other five test substances were discarded as of too little value to compensate for the time required for their use.

The morphology of this group differentiates it from the other cheese organisms.

Two hundred and forty-nine cultures of the *Bacterium casei* group have been submitted to the tests, which differentiated them into 12 strains, as given in Table III. It was necessary to combine this large number of strains into varieties, in order to make the classification workable in the study of the cheese problem.

TABLE III.—*Types of Bacterium casei as differentiated by biochemical tests*

Strain No.	Number of cultures studied.	Production of acid in broth containing—				
		Lactose.	Salicin.	Sucrose.	Mannit.	Inulin.
1	5	−	−	−	−	−
2	117	+	−	−	−	−
3	22	+	+	−	−	−
4	12	+	−	+	−	−
5	18	+	+	+	−	−
6	7	+	−	−	+	−
7	14	+	+	−	+	−
8	7	+	−	+	+	−
9	28	+	+	+	+	−
10	1	+	−	+	−	+
11	12	+	+	+	−	+
12	7	+	+	+	+	+

Strain 2, which ferments lactose alone and includes almost one-half of the cultures studied, naturally formed one variety. With it was included the five cultures which failed to ferment lactose in broth cultures, although they fermented lactose in milk cultures. The second variety includes all cultures which ferment lactose, and one, two, or three of the test substances—salicin, sucrose, and mannit. A third variety is characterized by its ability to ferment inulin. This variety also ferments lactose and one, two, or three of the substances, salicin, sucrose, and mannit. The three varieties have been designated "a," "b," and "c" in Table IV.

TABLE IV.—*Varieties of Bacterium casei found in cheese*

Variety No.	Production of acid in broth containing—				
	Lactose.	Salicin.	Sucrose.	Mannit.	Inulin.
a	+	−	−	−	−
b	+	±	±	±	−
c	+	±	±	±	+

There is a great variation in the amount of acidity formed in milk by the various cultures of a given strain. In Table V are given the range of maximum percentages of acidity found within the strain. The average percentage of acidity formed by the cultures increases with the ability to break down the more complex test substances. The average percentages for the three varieties are given in Table VI. The acid has been calculated as lactic acid.

TABLE V.—*Range of percentages of acidity formed by the strains of Bacterium casei*

Strain No.	Number of cultures studied.	Range of maximum percentages of acidity.	Strain No.	Number of cultures studied.	Range of maximum percentages of acidity.
1	1	0.68	7	12	0.91 to 1.97
2	60	.10 to 1.52	8	6	.94 to 1.69
3	8	.73 to 2.37	9	14	1.38 to 1.80
4	12	.42 to 1.50	10	1	.97
5	16	.47 to 1.97	11	6	.86 to 1.60
6	5	.85 to 1.35	12	1	1.74

TABLE VI.—*Variation in the average percentage of acidity formed by the varieties of Bacterium casei*

Variety No.	Number of cultures titrated.	Average percentage of acidity.
a	60	0.75
b	72	1.03
c	10	1.23

The statement was made in a previous publication (Hastings, Evans, and Hart, 1912) that the width of the cells of the lactic bacilli in a pure culture appear to be fairly constant, but that in come cultures the individual cells are all slender, whereas in other cultures they are all comparatively thick. Measurements have been made of the width of the cells in many cultures. The averages for the three varieties are given in Table VII.

TABLE VII.—*Variations in the average width of the cells of the varieties of Bacterium casei*

Variety No.	Number of cultures measured.	Average width of the cells in microns.
a	25	1.06
b	22	.81
c	3	.53

Thus, it is shown that the width of the cells decreases with their ability to attack the more difficultly fermentable substances and with their ability to produce higher percentages of acidity. The differences in width are so great that with a glance at a microscopic field of a culture containing cells of either extreme in width, the culture may be classed as a high or a low acid-producing variety.

DISTRIBUTION OF THE GROUPS AND VARIETIES OF CHEESE ORGANISMS IN NORMAL CHEDDAR CHEESE

Twenty-one raw-milk cheeses have been analyzed bacteriologically and the cultures classified in the manner described above. Some of the cheeses were analyzed only once or a few times. In Table VIII are given the percentages of the different varieties which were found in a series of 16 cheeses. These were the best of a large number of cheeses which had been sent for scoring to the Dairy Department of the University of Wisconsin from cheese factories in different parts of the State. They represent a high quality of Wisconsin cheese.

It will be observed that almost every variety of each of the four groups of cheese organisms is represented in the 16 cheeses in sufficiently large numbers to be influential in the development of flavor. It is not to be supposed that representatives of all of the varieties occurring in a cheese can be isolated each time the cheese is submitted to analysis. Particularly in the case of those cheeses which were analyzed only once, other forms than those shown by the data must have been present. For this reason Table VIII as a whole rather than the data from any individual cheese should be considered in drawing conclusions as to the types of organisms present in normal cheese. The figures of the table indicate that the flora of a normal Cheddar cheese is varied and consists of representatives of each of the four groups of cheese organisms.

TABLE VIII.—*Bacterial content of normal raw-milk cheeses*

Cheese No.	Age.	Bacteria per gram (plate cultures).	Variety of Bacterium lactis acidi.			Variety of Bacterium casei.			Variety of streptococci.				Variety of micrococci.			Bacteria per gram (dilution cultures).	Variety of Bacterium casei.	
			a	b	d	a	b	c	a	b	c	d	a	b	c		a	b
	Days.		*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*		*P.c.*	*P.c.*
33....	30	35,000,000	....	....	100	....	....	....	....	....	....	....	....	....	....	100,000,000	[a]+	....
35....	30	8,000,000	....	63	....	25	....	....	....	....	....	12	....	....	....	10,000,000	....	[a]+
44....	32	4,000,000	25	....	....	....	....	12	....	....	....	25	....	38	....	10,000,000	....	[a]+
36....	28	10,500,000	40	....	....	10	20	....	....	30	....	....	....	....	....	1,000,000,000	....	[a]+
49....	32	3,200,000	....	....	71	29	....	....	....	....	....	...	[illegible]	[illegible]	..	10,000,000	....	[a]+
38....	48	2,000,000	12	....	13	12	....	....	....	25	13	[illegible]	[illegible]	[illegible]	[illegible]	1,000,000	....	....
45....	38	55,000,000	67	....	....	....	22	....	....	....	..	[illegible]	[illegible]	[illegible]	[illegible]	100,000,000	....	[a]+
50....	24	7,000,000	30	....	50	....	....	....	10	....	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0,000,000	....	....
53....	30	8,000,000	70	....	....	....	....	....	....	....	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	,000,000	....	[a]+
63....	30	3,500,000	10	20	50	....	10	....	....	10	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	000,000	....	[a]+
5....	45	18,500,000	....	....	....	....	90	....	....	..	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	00,000	....	[a]+
	52	36,000,000	11	....	....	....	67	....	11	.	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	00,000	....	[a]+
	68	11,000,000	20	....	....	....	80	....	....	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0,000	....	[a]+
13....	37	28,000,000	50	....	....	....	33	....	....	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[a]+	....
	43	25,000,000	63	....	....	13	....	....	12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	59	3,200,000	....	....	30	10	48	....	...	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
16....	39	10,000,000	14	....	....	58	....	....	...	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	46	5,000,000	14	....	....	....	86	....	..	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	62	4,500,000	....	....	....	....	100	....	..	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
17....	18	12,000,000	90	....	....	....	....	....	..	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	25	15,000,000	50	....	....	....	10	....	..	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	41	11,500,000	10	....	....	80	10	....	..	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
130....	16	600,000,000	....	....	17	83	....	....	..	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	32	125,000,000	....	78	....	....	....	....	..	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
131....	14	38,000,000	....	76	....	....	11	....	...	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	30	120,000,000	....	11	....	88	....	....	...	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	....	....

[a] +=Found pr

A more comprehensive study was made of raw-milk cheese No. 17 R. Analyses were made during the making and at intervals throughout the ripening period until the cheese was 8 months old. The results of this study are given in Table IX.

TABLE IX.—*Bacterial content of raw-milk cheese No. 17 R*

Age.	Bacteria per gram (plate cultures).	Variety of Bacterium lactis acidi.				Variety of Bacterium casei.		Variety of streptococci.			Variety of micrococci.		Bacteria per gram (dilution cultures).	Variety of Bacterium lactis acidi.			Variety of Bacterium casei.	
		a	b	c	d	a	b	b	c	d	a	b		a	b	d	a	b
Days.		*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*	*P.c.*		*P.c.*	*P.c.*	*P. c.*	*P.c.*	*P. c.*
Curd.	100,000,000	....	30.0	....	70.0	....	....	....	....	....	....	....	100,000,000	....	....	99.999	....	0.001
4	70,000,000	10.0	....	....	40.0	....	....	50.0	....	....	....	....	100,000,000	....	....	......	....	.....
8	900,000,000	....	88.8	....	....	....	....	....	11.2	....	....	....	1,000,000,000	....	....	9.99	....	.01
15	120,000,000	....	....	90.0	....	....	10	....	....	....	....	....	1,000,000,000	....	99.0	......	....	1.00
22	240,000,000	....	87.5	....	....	12.5	....	....	....	....	....	....	100,000,000	99.0	....	......	1.0	.....
29	280,000,000	66.3	....	....	22.2	....	....	....	....	11.2	0.3	....	1,000,000,000	99.0	....	......	1.0	.....
36	400,000,000	....	10.0	....	80.0	....	....	....	....	....	10.0	....	100,000,000	....	....	90.90	9.1	.....
43	160,000,000	43.0	....	56.3	....	....	....	....	.1	.2	.1	0.3	100,000,000	90.9	....	......	9.1	.....
50	200,000,000	....	70.0	....	....	....	....	17.5	....	....	12.5	....	100,000,000	90.9	....	......	9.1	.....
57	100,000,000	....	....	....	76.5	....	....	....	....	20.0	3.5	....	....	....	....	......	....	.....
64	80,000,000	....	88.8	....	....	....	....	....	....	10.0	1.2	....	100,000,000	....	90.9	......	9.1	.....
71	100,000,000	35.1	....	....	41.7	20.0	....	....	....	....	3.2	....	100,000,000	....	90.9	......	9.1	.....
89	32,000,000	86.9	....	....	....	....	....	10.0	....	....	3.1	....	100,000,000	99.0	....	......	1.0	.....
172	7,000,000	....	22.0	....	67.0	....	....	....	....	....	11.0	....	100,000,000	....	....	90.9	9.1	.....
245	12,000,000	....	....	....	....	20.0	80	....	....	....	....	....	100,000,000	....	....	50.0	50.0	.....

Where very small percentages appear in the columns of the coccus groups the figures were obtained by plating upon an agar made with cheese extract. Upon this medium the lactic-acid groups of organisms form very small colonies, but the cocci, particularly the micrococci, develop large colonies and can be easily differentiated and isolated from plates which are very thickly seeded.

Cheese No. 17 R was a good Cheddar cheese, with the characteristic flavor well developed when 1 month old. The cheese remained in good condition until the last analysis, with an increasing sharpness of taste which is common to well-matured cheese. The flora is considered typical of a good Cheddar cheese. The data confirm those presented in Table VIII. Twelve of the fifteen varieties of cheese organisms representing the four groups were isolated from this cheese. The data presented in Tables VIII and IX show that besides the *Bacterium lactis acidi* group, which has long been known to be important in cheese ripening, and the *B. casei* group, which was shown (Hastings, Evans, and Hart, 1912) to be constantly present in cheese in numbers almost as great as the former group. Coccus forms are also constantly present, and although they are fewer in number than the lactic-acid-forming groups they occur in sufficient numbers to affect flavor development.

Other evidence of the constant presence of several varieties of bacteria in the cheese flora is found in the gradual decline in acid production, as the dilutions increase, in milk inoculated with the cheese emulsion. In the present study of cheese flora, several hundreds of series of titrations have been made of the milk cultures which have been mentioned as a part of the routine method of cheese analysis. There is in nearly every series a general decline as the emulsion used for the inoculation becomes more and more dilute. The two series presented in Table X illustrate the point. The titrations were made after 28 days incubation at 35° C. and the acidity calculated as lactic acid.

TABLE X.—*Decline in acidity with increasing dilutions of cheese emulsion used for inoculation of milk cultures*

Cheese No. 312 C.		Cheese No. 309 C.	
Dilution.	Percentage of acidity.	Dilution.	Percentage of acidity.
1:100,000	1.60	1:10,000	1.54
1:1,000,000	1.55	1:100,000	1.45
1:10,000,000	1.22	1:1,000,000	1.50
1:100,000,000	.78	1:10,000,000	1.18
1:1,000,000,000	.67	1:100,000,000	1.00
1:10,000,000,000	(a)	1:1,000,000,000	(a)

[a] No growth.

The lower percentage of acidity with every tenfold dilution indicates that in each dilution there were present certain varieties of organisms in such small proportions that they did not occur in the next higher dilution. Out of 345 series of titrations the decline was without a break in 56 per cent of the cases; in 37 per cent of the cases there was a general decline, but with one or more breaks; in only 7 per cent of the cases was this decline not apparent.

THE VARIATION IN CHEESE FLORA

It is evident from Tables VIII and IX that the normal flora of Cheddar cheese is varied, with varieties of all four groups of cheese organisms in numbers ranging from hundreds of thousands to billions per gram of cheese. The flora in two equally good cheeses will contain each of the four groups of cheese organisms, but will differ as to the varieties present. There will also be a variation in the proportion of organisms of any single group. It does not appear that it is essential to good flavor to have any single variety present in very high proportions. One cheese may contain a given variety in high proportions, whereas another cheese may have this variety in such low proportions that it does not make its appearance in a bacteriological analysis. To illustrate: Streptococcus "b," which formed 30 per cent of the flora of cheese No. 36 (Table VIII), was isolated from only 6 of the 17 cheeses whose flora are presented in Tables VIII and IX.

This variation of flora, with a constancy to the general type of growth, is to be expected under any ecological conditions determined by natural circumstances, for the ripening of Cheddar cheese must be regarded as a spontaneous fermentation, limited to certain groups of bacteria by the conditions obtaining in the cheese mass and subject to variation within the groups by accident of inoculation before or during the cheese making, or by slight variations in the raw material or in the method of manufacture, which enable one variety or the other to gain an ascendancy.

SYMBIOTIC RELATION OF CHEESE ORGANISMS

The complex chemical changes taking place in a mixed culture of bacteria have usually been regarded as the result of the combined activity of the individual properties of each of the several species when grown alone, with these properties accelerated in some cases by the associated action.

Among the many cultures of cheese organisms inoculated into the carbohydrate media, it was observed that when by accident the culture was contaminated with other cheese organisms through failure of a pure isolation, there appeared to be a high ability to attack the more complex substances. Accordingly many inoculations of cheese cultures were made into the test media in various combinations, to determine whether associative action would give new properties to the cultures concerned. In this experiment the test for acid production was the reddening of litmus paper. In many cases acid was produced from a given substance by the associated action, when neither culture working alone would give such a reaction. A few examples are given in Table XI to show the results of these inoculations.

TABLE XI.—*The effect of the associative action of cheese organisms in breaking down test substances*

Organism.	Production of acid in broth containing—					
	Lactose.	Salicin.	Sucrose.	Glycerin.	Mannit.	Inulin.
Bacterium lactis acidi, a	+	−	−	−	−	....
Bacterium casei, a	+	−	−	....	−	−
Bacterium casei, b	+	+	−	....	+	−
Streptococcus, b	+	−	−	−	−	....
Streptococcus, c	+	+	+	−	−	....
Micrococcus, b	+	−	−	−	−	....
Bacterium lactis acidi, a; Bacterium casei, a	+	−	+	−	−	−
Bacterium lactis acidi, a; Bacterium casei, b	+	+	+	−	+	−
Bacterium lactis acidi, a; Streptococcus, b	+	+	+	−	−	−
Bacterium lactis acidi, a; Streptococcus, c	+	+	+	+	−	−
Bacterium casei, a; Bacterium casei, b	+	+	+	−	+	−
Bacterium casei, a; Streptococcus, b	+	−	+	−	−	−
Bacterium casei, a; Streptococcus, c	+	+	+	+	+	−
Bacterium casei, a; Micrococcus, b	+	+	+	−	−	−

Further inoculations were made into broth containing the test substances to determine the quantitative effect of this associative action. The results of some of these inoculations are given in Table XII, in which the acidity is expressed as lactic acid.

TABLE XII.—*The effect of the associative action of cheese organisms in the production of acid*

Organism.	Medium.					
	Salicin.		Sucrose.		Mannit.	
	Individual action.	Associative action.	Individual action.	Associative action.	Individual action.	Associative action.
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Bacterium lactis acidi, a	0.00	0.39	0.00	0.00	0.00	0.00
Bacterium casei, a	.00		.00		.00	
Bacterium lactis acidi, a	.00	.32	.00	.00	.00	.00
Streptococcus, b	.00		.00		.00	
Bacterium lactis acidi, b	.00	.37	.00	.28	.00	.00
Bacterium casei, a	.30		.00		.00	
Bacterium lactis acidi, b	.00	.32	.00	.51	.00	.35
Micrococcus, c	.39		.44		.00	
Bacterium lactis acidi, d	.00	.00	.00	.17	.00	.28
Bacterium casei, a	.00		.00		.00	
Bacterium lactis acidi, d	.00	.21	.00	......	.00	.31
Micrococcus, b	.00		.00		.33	

The data show that considerable quantities of acid can be formed in mixed cultures even though the individual cultures are unable to ferment the substance in question. In the *Bacterium lactis acidi* group, 13 positive reactions were obtained and determined by titration in cultures in which a member of this group was mixed with a representative of one of the other three groups of cheese organisms, while 23 cases were negative. In the entire 36 tests none of the organisms was able to ferment the substance used when working alone. The same symbiotic relations were exhibited by the *B. casei* group when a member of this group was grown together with a representative of one of the other groups of cheese organisms or with another variety of the same group. (See Table XI.) Thus, it appears not only that the influence upon each other of the cheese organisms is beneficial in enhancing the individual powers in many cases but also that the symbiosis enables the cheese organisms to attack other food substances than any one species working alone would be able to utilize.

The fact that the normal flora of Cheddar cheese consists of a number of varieties of the four groups of cheese organisms and that these exert upon one another a decided influence in their chemical activities is most important in considering the cheese-ripening problem.

BACTERIAL FLORA OF PASTEURIZED MILK CHEESE

The influence of this varied flora upon the production of flavor is emphasized in a study of the flora of pasteurized-milk cheese. During the pasteurization approximately 99 per cent of the bacteria of the milk are killed (Sammis and Bruhn, 1912); then there are added, in the form of a starter, organisms belonging to the *Bacterium lactis acidi* group. But instead of ripening the milk until the desired acidity for cheese making is obtained—the usual procedure in the making of raw-milk cheese—a weak solution of hydrochloric acid is added to make up the required acidity. This is necessary in preparing pasteurized milk for cheese making.

It is evident that the initial bacterial flora of the cheese made from pasteurized milk must differ from the flora of a raw-milk cheese of the same age, for in the latter all of the types of organisms making up the varied flora of milk have had an opportunity to develop without any other restraint than that of their symbiotic relations with the associated types.

In studying the flora of cheese in respect to its influence in flavor production many bacterial analyses have been made of pasteurized-milk cheese in every stage of the ripening process. The cultures isolated have been studied in the same manner as those from raw-milk cheese and have been classified accordingly. In Tables XIV and XV are presented the results of the analyses of two pasteurized-milk cheeses, Nos. 20 and 21, to which pure-culture starters were added. To cheese No. 20 (Table XIII), there was added 0.75 per cent of a pure-milk culture of *Bacterium lactis acidi*, b. In striking contrast to the varied flora of raw-milk cheese, as presented in Tables VIII and IX, it will be observed that the bacterial flora of this cheese consisted almost entirely of the one variety of organism, *B. lactis acidi*, b, which was added to the milk for a starter. Only once was another variety of this group isolated.

TABLE XIII.—*Bacterial content of pasteurized-milk cheese No. 20 to which was added 0.75 per cent of a culture of Bacterium lactis acidi, b*

Age.	Plate cultures.							Dilution cultures.			
	Bacterial content.	Variety of Bacterium lactis acidi.		Variety of streptococci.	Variety of micrococci.		Contamination.	Bacterial content.	Variety of Bacterium lactis acidi.	Variety of Bacterium casei.	
		a	b	d	a	b			b	a	b
Days or state.		*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*	*P. ct.*		*P. ct.*	*P. ct.*	*P. ct.*
Milk..	9,600,000	....	99.70	......	......	......	0.30	100,000,000	....	....	0.0001
Curd .	20,000,000	....	98.00	......	......	......	2.00	100,000,000	99.9999	0.0001	....
6....	450,000,000	....	100.00	......	....	....	....	1,000,000,000	....	....	....
14....	175,000,000	....	96.80	......	0.2	......	3.00	100,000,000	100.00	....	....
22....	175,000,000	....	98.30	......	......	......	1.70	100,000,000	100.00	....	....
30....	400,000,000	....	95.75	......	......	......	4.25	10,000,000,000	....	....	....
38....	300,000,000	28.2	70.50	......	......	......	1.30	1,000.000,000	....	....	....
47....	1,650,000,000	....	100.00	......	......	......	....	1,000,000,000	99.00	1.0000	....
58....	65,000,000	....	99.77	0.04	......	0.19	....	....	....	....	....
72....	170,000,000	....	100.00	......	......	......	....	100,000,000	90.90	....	9.1000

One variety of the Streptococcus group was isolated once, and two varieties of the Micrococcus were isolated, each only once, in small percentages. The small percentages of cocci were isolated from cheese-agar plates in the same manner as was followed in the analysis of the raw-milk cheese presented in Table IX. Cheese No. 20 was quite badly contaminated with a liquefying coccus which infected each of the three vats of milk made up separately into cheese. The contamination is given in a separate column. The data from the dilution cultures show *Bacterium lactis acidi*, b, as the predominating organism of the cheese at every examination. The *B. casei* group developed slowly in this cheese. It was present in exceedingly small percentage during the first few days. Unfortunately the dilutions were made too high, so that this group did not appear in the dilution cultures in the analyses made between the sixth and forty-seventh days. At the forty-seventh and seventy-second days there were present 1,000,000 bacteria of this group per gram of cheese.

TABLE XIV.—*Bacterial content of pasteurized-milk cheese No. 21 to which was added 0.75 per cent of a culture of Bacterium lactis acidi, d*

Age.	Plate cultures.				Dilution cultures.			
	Bacteria content.	Variety of B. lactis acidi, d.	Variety of micrococci.		Bacterial content.	Variety of B. lactis acidi, d.	Variety of Bact. casei.	
			a	b			a	b
Days or state.		*Per cent.*	*Per ct.*	*Per ct.*		*Per ct.*	*Per ct.*	*Per ct.*
Milk					10,000,000	100.00		
Curd	250,000,000	100.00			100,000,000			
5	270,000,000	100.00			100,000.000	99.999	0.001	
13	1,120,000,000	100.00			1,000,000,000	100.00		
21	1,200,000,000	100.00			1,000,000,000			
29	250,000,000	100.00			1,000,000,000	100.00		
37	1,400,000,000	99.99	0.01		1,000,000,000			
46	1,200,000,000	99.99	.01		1,000,000,000	99.90	.100	
57	1,000,000,000	99.94	.02	0.04				
71	1,480,000,000	100.00			1,000,000,000	99.90		0.1

Cheese No. 21 (see Table XIV) received three-fourths per cent of *Bacterium lactis acidi*, d, as a starter. A study of the pure cultures isolated from this cheese shows that this variety composed practically 100 per cent of the cheese organisms, as determined by plate cultures. No other variety of *Bacterium lactis acidi* and no variety of the Streptococcus group appeared. Small percentages of micrococci were isolated on the thirty-seventh, forty-sixth, and fifty-seventh days. The pure cultures isolated from the dilution flasks showed only *B. lactis acidi*, d, and a slowly increasing number of *B. casei*, which reached 1,000,000 of these bacteria per gram of cheese on the forty-sixth day.

The data in Tables XIII and XIV illustrate well the facts which are demonstrated by a study of the results of many analyses of 14 pasteurized-milk cheeses. In some of these cheeses a mixture of several pure cultures was used as a starter. Usually every type which was added to the cheese was isolated in considerable percentages in some one or more of the analyses. Thus, it has been shown that the flora of pasteurized-milk cheese consists almost entirely of the organisms which are introduced in the starter, with a small percentage of micrococci and steadily increasing numbers of *Bacterium casei.*

RELATION OF THE VARIOUS TYPES OF CHEESE ORGANISMS TO FLAVOR PRODUCTION

Since it has been shown that in pasteurized-milk cheese the bacterial content is practically limited to the varieties which are added during the making, together with small percentages of micrococci, and a development of the *Bacterium casei* group similar to that in a raw-milk cheese, although it is usually more or less retarded, it is possible to gain some knowledge of the influence of the different varieties in pure culture upon flavor production.

A normal raw-milk cheese of the Cheddar type will, after a few days' ripening, begin to develop a delicate flavor which is characteristic of this type of cheese. This flavor becomes intensified as the cheese matures, and after ripening for a number of weeks, depending upon the temperature at which the cheese is kept, it acquires a pungent taste which also intensifies with continued ripening.

A pasteurized-milk cheese made with a commercial starter will never develop the Cheddar flavor which characterizes a young raw-milk cheese of this type, but it does develop an acid flavor which is pleasant to the taste of many people. If the cheese is kept for several months, the acid flavor disappears and the biting taste common to well-matured raw-milk cheese becomes the characteristic flavor.

THE RÔLE OF BACTERIUM CASEI IN FLAVOR PRODUCTION

The floras of the two types of cheese, which differ essentially during the first few weeks of ripening, become more and more alike as the cheese matures. As shown in Table XV, *Bacterium casei* develops in pasteurized-milk cheese in as large numbers as in raw-milk cheese. It is reasonable, therefore, to ascribe to this group of organisms the development of the pungent flavor common to the two types of well-matured cheese.

In Table XV are given the numbers of *Bacterium casei* in a raw-milk and in a pasteurized-milk cheese made the same day from the same lot of milk. Until the forty-second day the raw-milk cheese contains about 10 times as many of the *B. casei* as the pasteurized-milk cheese, but after the forty-second day the cheeses contain about equal numbers of these organisms.

Cheeses Nos. 307 C and 307 illustrate the *Bacterium casei* content found in 12 similar cheeses—6 of the raw milk and 6 of the pasteurized milk—

made on successive days. The two cheeses selected were among the best flavored of the lot and are typical cheeses of the raw-milk and pasteurized-milk types. The Cheddar flavor was already developing in the raw-milk cheese when first examined for flavor at 18 days. This flavor increased in strength until the ninety-eighth day, when the pungent taste became evident. As the cheese aged, the sharpness became more and more intense. In the pasteurized-milk cheese no other flavor than a sour taste was apparent until the cheese was about 7 months old, when it possessed a mild, sharp taste.

TABLE XV.—*The correlation of the development of the Bacterium casei group and the pungent taste in raw-milk and pasteurized-milk cheese*

Age.	Cheese No. 307 C (raw milk).		Cheese No. 307 (pasteurized milk).	
	Number of individuals of Bacterium casei per gram of cheese.	Flavor.	Number of individuals of Bacterium casei per gram of cheese.	Flavor.
Days.				
2	10, 000			
3	10, 000		10, 000	
4	100, 000		10, 000	
5	100, 000		10, 000	
7	10, 000, 000		1, 000, 000	
9	100, 000, 000		1, 000, 000	
11	1, 000, 000		1, 000, 000	
14	10, 000, 000		10, 000, 000	
18	10, 000, 000	Cheddar, developing	1, 000, 000	Cottage-cheese.
21	10, 000, 000		100, 000	
29	100, 000, 000		10, 000, 000	
36	100, 000, 000		10, 000, 000	
42	10, 000, 000		10, 000, 000	
49	10, 000, 000	Strong Cheddar		Only a sour taste.
56	10, 000, 000		1, 000, 000	Do.
71	10, 000, 000	Strong Cheddar	100, 000, 000	Clean, sour taste; no Cheddar.
98	10, 000, 000	Strong Cheddar; getting sharp.	10, 000, 000	Do.
108	10, 000, 000		100, 000, 000	Do.
150	1, 000, 000	Strong Cheddar; sharp	10, 000, 000	Do.
218	10, 000, 000	Extremely sharp	1, 000, 000	Acid flavor has disappeared. Mild sharpness.
311		Intensely sharp		Good cheese. Sharp.

Jensen (1904, p. 356) has shown that the *Bacterium casei* group is active in breaking down the casein of milk to which calcium carbonate is added and maintains that the casein is not peptonized, but is split directly into monoamino acids.

Van Slyke and Hart (1903) have shown that there is a constantly increasing percentage of monoamino acids in ripening Cheddar cheese. At three months more than one-third of the water-soluble nitrogenous compounds is in this form, and later a much larger percentage. The evidence seems to point to the fact that in raw-milk Cheddar cheese and in the pasteurized-milk cheese the *Bacterium casei* group common to both of these types of cheese is responsible for the biting taste which is characteristic of the well-ripened cheese.

Jensen has explained the action of the *Bacterium casei* group in producing amino acids as due to an "endoerepsin" set free by the dead cells rather than to the activity of the living bacteria, because he found the greatest amount of amino acids formed after the bacteria were for the most part dead (Jensen, 1912).

Whatever may be the condition in the Emmenthaler cheese which Jensen studied, this explanation for the activity of the *Bacterium casei* group in Cheddar cheese is not necessary. It appears to be certain that the living bacteria were active throughout the ripening period in this type of cheese. Cheese No. 17 R (Table IX) and cheese No. 307 C (Table XV) both demonstrated this fact. In cheese No. 17 R there were 10,000,000 living bacteria of the *B. casei* group per gram of cheese when 8 months old; cheese No. 307 C contained a similar number at 7 months. These bacteria did not appear to be living in a latent condition, for all three varieties of *B. casei* readily grew upon an agar made with only an extract of the cheese for a food substance. Every time an analysis was made of cheese No. 17 R such an agar was prepared on the day the analysis was made, using for the cheese agar a part of the plug which served for bacteriological analysis. Thus, the cheese organisms were submitted for development upon the same food substance which served them in the cheese itself.

The result of the bacterial count upon this cheese agar was only slightly smaller than the total count upon the casein agar. Every variety of cheese organisms grew upon this medium. On the eighty-ninth day, when 32,000,000 organisms per gram of cheese developed colonies on the casein agar and of the 10 colonies isolated none belonged to the *Bacterium casei* group, 20,000,000 colonies developed upon the cheese agar, of which 20 per cent, or 4,000,000 bacteria per gram, belonged to the *B. casei* group. The flask dilutions on that same date showed 1,000,000 bacteria of this group per gram of cheese. Thus, it is shown that several millions of living organisms of the *B. casei* group are present in a normal Cheddar cheese 3 months of age, and the cheese itself provides suitable food for development. It is most probable that under these conditions the living bacteria are active in the cheese.

INFLUENCE OF BACTERIUM CASEI IN STARTERS FOR PASTEURIZED-MILK CHEESE

Many experimental pasteurized-milk cheeses have been made with pure-culture starters, to determine their influence upon the production of flavor.

In the first series some variety of *Bacterium casei* was added to a number of the cheeses, together with the *B. lactis acidi*. When variety "a" was added, there was a tendency for the cheese to become "acid injured"—strongly acid, friable, and opaque. The use of variety "b" as a starter was more likely to bring about this condition. When variety "c" was used, the cheese was almost certain to be ruined by the acid before it was a month old. This variety was isolated only once from the 21 normal raw-milk cheeses which have been studied with reference to the varieties; and then there were present less than 500,000 bacteria per gram of cheese. *B. casei*, c, has been isolated, however, from three raw-milk cheeses which had a sour taste rather than the Cheddar flavor. Therefore this variety can not be regarded as a necessary organism in normal ripening. It is likely that it never occurs in large numbers in the young cheese without causing injury. *B. casei*, varieties "a" and "b," are about equally distributed in normal Cheddar cheese, where they usually occur together and perform an active part in the ripening changes. It has been noted that the *B. casei* groups develop gradually in the pasteurized-milk cheese, as they do in the raw-milk cheese, although usually more slowly. The introduction of this group as a starter, however, resulting in abnormally large numbers of *B. casei* in the early ripening period, is detrimental to the cheese.

In subsequent series of experiments with pasteurized-milk cheese this group of organisms was never added to the starter.

INFLUENCE OF BACTERIUM LACTIS ACIDI UPON FLAVOR PRODUCTION

When *Bacterium lactis acidi*, a or b, is used for a starter in pure culture in pasteurized-milk cheese, an acid taste is produced which is characteristic of the ordinary pasteurized-milk cheese made with the use of a commercial starter. No suggestion of a Cheddar flavor is ever obtained. If *B. lactis acidi*, d, is added to the milk, there is almost always produced a peculiar flavor, which, as it intensifies with continued ripening, becomes decidedly bitter. Out of 14 experimental cheeses in which 0.75 per cent, the ordinary quantity used for a starter, of a pure culture of this variety was used or in which a mixture of several pure cultures was used with *B. lactis acidi*, d, in large proportions, the unpleasant flavor has developed some time between the fourth and fourteenth weeks. Usually the cheese becomes bitter by the time it is 2 months old. In only one instance has the bitterness failed to develop before the fourteenth week. The figures in Tables VIII and IX show that *B. lactis acidi*, d, was isolated from normal raw-milk cheese with sufficient frequency that it may be concluded that it is always present in this type of cheese in large numbers. It no doubt contributes to the characteristic Cheddar flavor under the conditions obtaining in a normal cheese. But it is certain that a large amount of this variety is not suitable for use in the starter for pasteurized-milk cheese.

INFLUENCE OF THE COCCUS GROUPS IN THE PRODUCTION OF FLAVOR

From the frequency of occurrence of all four varieties of the streptococci in normal Cheddar cheese in percentages ranging as high as 50, as shown in Tables VIII and IX, it is most certain that this group is active in the ripening changes. The by-products of this group of organisms are entirely different from those of the *Bacterium lactis acidi* group. Instead of a large quantity of lactic acid, with small quantities of other acids, as formed by the latter group, the streptococci produce no lactic acid, but produce large amounts of acetic acid, with smaller percentages of propionic, butyric, and caproic acids (Hart, Hastings, Flint, and Evans, 1914). The streptococci were also shown to produce small amounts of ammonia, a by-product not found in *B. lactis acidi* cultures. It is therefore to be expected that the large numbers of streptococci present in Cheddar cheese have a decided influence upon flavor development. The influence of individual cultures of this group in the ripening of pasteurized-milk cheese will be discussed later.

Bacteria of the Micrococcus group were isolated from normal Cheddar cheese in percentages as high as 40. It does not appear that this group of organisms is of primal importance in the production of Cheddar flavor, however, for they have been found commonly in pasteurized-milk cheese in numbers comparable with those found in the raw-milk cheese with well-developed flavor. When added to the cheese in large percentages of the starter, a bitterness is always produced within a few weeks.

EXPERIMENTS IN THE PRODUCTION OF DESIRABLE FLAVORS IN PASTEURIZED-MILK CHEESE

The study of the bacterial content of normal Cheddar cheese has demonstrated the fact that the flora is varied and is made up of several varieties of all four groups of cheese organisms. Therefore, in the attempt to prepare starters which might develop Cheddar flavors in the pasteurized-milk cheese it appeared reasonable to mix pure cultures together for the cheese inoculation, choosing the varieties most frequently found in Cheddar cheese with the well-developed flavor. Many experimental cheeses have been made with the use of such starters, the mixtures being made up of from two to nine pure cultures in various combinations and varying percentages, in order to determine which of the varieties in addition to *Bacterium lactis acidi* might improve the acid flavor of the pasteurized-milk cheese.

The difficulties in preparing a starter which will reinstate in pasteurized milk a flora which will simulate raw milk well ripened for cheese making are apparent, even though the relative percentages of each group and variety were better known.

Out of 20 experimental cheeses which were inoculated with *Bacterium lactis acidi*, a or b, together with one of the other varieties of this group or some variety of the coccus groups or with some mixture of these pure cultures, there was usually obtained a better flavor in the young cheese than in the control cheese inoculated with a pure culture of *B. lactis acidi*, a or b, alone. But by the time the cheese was well ripened a bitterness had usually developed. It was observed that, as in the *B. lactis acidi* group, the coccus cultures with a high ability to ferment the more complex test substances were likely to produce bitterness when inoculated in large percentages. The fact that some of these cultures with high fermenting ability were included in almost all of the mixtures is thought to account for the development of bitterness in this series of experimental cheeses. Nevertheless these organisms may participate in the production of a good Cheddar flavor under the conditions for their development in the raw-milk cheese. In only one cheese of this series was a semblance of a Cheddar flavor obtained. This cheese was inoculated with the following mixture: *B. lactis acidi*, b, 42 per cent; *B. lactis acidi*, d, 48 per cent; Streptococcus, c, and Micrococcus, b, each 5 per cent. At three months there was an unmistakable resemblance to a Cheddar flavor. At four months the cheese scored as high as 94.6 per cent when examined by experts. Thus, the Cheddar flavor was obtained with this combination of cultures, most of which produced bitterness when used individually.

It was observed that several cheeses of this series, to which there was added a considerable percentage of Streptococcus, b, developed a pleasant flavor, an improvement upon the acid flavor of *Bacterium lactis acidi* alone, although it was not the Cheddar flavor. A third lot of experimental pasteurized-milk cheeses was made, in which the value of Streptococcus, b, in various proportions, together with *B. lactis acidi*, b, was tested. Three vats of cheese were made on each of three successive days. In series A, 50 per cent or more of the starter was a culture of Streptococcus, b; in series B this organism made up 33⅓ per cent or less of the starter. In series C the starter was a pure culture of *B. lactis acidi*, b. In Table XVI the scores for the cheeses are given, as determined by expert judges. The average score of the three judges for each cheese is given, 100 being perfect.

TABLE XVI.—*The effect upon flavor development of the use of various percentages of Streptococcus, b, in the starter for pasteurized-milk cheese*

Cheese No.	Series A.		Series B.		Series C.	
	Inoculation.	Score.	Inoculation.	Score.	Inoculation.	Score.
30	Bacterium lactis acidi, b (50 per cent; Streptococcus, b (50 per cent).	90.4	Bacterium lactis acidi, b (66⅔ per cent); Streptococcus, b (33⅓ per cent).	92.7	Bacterium lactis acidi, b (100 per cent).	90.9
31	Bacterium lactis acidi, b (50 per cent); Streptococcus, b (50 per cent).	90.2	....do................	93.5	.....do...............	91.7
32	Bacterium lactis acidi, b (50 per cent); Streptococcus, b (50 per cent).	94.0	Bacterium lactis acidi, b (75 per cent); Streptococcus, b (25 per cent).	93.8	.....do...............	91.0
	Average...............	92.2	....	93.3	....	91.2

It will be observed that for every day's make the cheese of series B, with the smaller percentages of Streptococcus, scored higher than series C, with the pure *Bacterium lactis acidi* starter. Series A, with the larger percentages of Streptococcus, scored higher than series C in two out of the three cheeses. Series B averaged 2.1 points higher than the series C, and series A averaged 1 point higher than series C.

The differences in flavor, however, were greater than the figures indicate, since the scores were made upon the commercial value of the cheese. The cheeses of series C had the acid flavor typical of a young pasteurized-milk cheese made with the ordinary commercial starter. The cheeses of series B had none of this acid flavor, but instead they possessed a mild flavor which was more agreeable to the taste of a number of persons knowing nothing of the experiment, to whose judgment the samples were submitted. All were agreed that the cheeses of series B lacked the acid taste. The cheeses of series A also differed greatly from those of the series C. They did not have the pleasant flavor of series B, however, but had an acid flavor essentially different from that of series C and inferior to the mild flavor of series B.

These experiments with the use of the Streptococcus, b, culture in starters demonstrate the fact that the addition of comparatively small percentages of this kind of culture brings about a decided difference in flavor, which is regarded as an improvement by all who have passed judgment in the matter. The larger percentages of streptococci give less desirable flavors than the smaller percentages. It seems probable that further experimentation with this and other cultures of Streptococcus, b, in combination with *Bacterium lactis acidi* may give results which will be of practical value in the improvement of flavors in pasteurized-milk cheese. And it does not seem unreasonable to hope that further experimentation with the *B. lactis acidi*, a or b, together with smaller percentages of Streptococcus, b, as the basis for a starter and with various combinations of very small percentage of the other cheese organisms, Cheddar flavors may be obtained in the pasteurized-milk cheese.

SUMMARY

(1) The organisms constantly found in Cheddar cheese in such numbers as to indicate they must function in the ripening process are included in four groups: First, the *Bacterium lactis acidi*; second, the *B. casei;* third, Streptococcus; fourth, Micrococcus.

(2) On the basis of the fermentation powers each of the four groups may be divided into a number of varieties.

(3) The distribution of the varieties of the four groups in Cheddar cheese prepared from raw milk has been studied, as has also been done with cheese prepared from pasteurized milk.

(4) The flora of raw-milk cheese is varied and consists of all the varieties into which the four groups were divided.

(5) The flora of pasteurized-milk cheese, with the exception of the *Bacterium casei* group, is dependent upon the flora of the starter.

(6) The *Bacterium casei* group is apparently responsible for the pungent taste that develops late in the ripening period of both raw-milk and pasteurized-milk cheeses. It is probable that growth of this group continues during the major part of the ripening period.

(7) The action of two or more organisms growing together is not the sum of their individual actions when growing alone. When growing together, they may attack substances that neither can attack alone, or they may produce a larger quantity of acid than the sum of the quantities that either can produce alone.

(8) When added to pasteurized milk, the organisms of the *Bacterium casei* group produce a sour taste in the cheese during the early part of the ripening period.

(9) No Cheddar flavor is obtained in pasteurized-milk cheese when the organisms of the *Bacterium lactis acidi* group alone are used as starters. The varieties that are able to ferment the more complex substances are likely to produce a bitter taste.

(10) Starters composed of both *Bacterium lactis acidi*, b, and Streptococcus, b, when added to pasteurized milk, improve the quality of the cheese. It does not seem unreasonable to hope that starters may be obtained that will give the characteristic Cheddar flavor to the cheese prepared from pasteurized milk.

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